A Kinome RNAi Screen in Drosophila Identifies Novel Genes Interacting with Lgl, aPKC, and Crb Cell Polarity Genes in Epithelial Tissues

Parsons, Linda M.; Grzeschik, Nicola A; Amaratunga, Kasun; Burke, Peter; Quinn, Leonie M; Richardson, Helena E

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Evaluation of the impact of tumor HPV status on outcome in patients with locally advanced unresectable head and neck squamous cell carcinoma (HNSCC) receiving cisplatin, 5-fluorouracil with or without docetaxel: A subset analysis of EORTC 24971 study

A. Psyrri[1], C. Fortpied[2], G. Koutsodontis[1], M. Avgeris[3], C. Kroupis[1], N. Goutas[1], J. Menis[2], L. Herman[2], L. Giurgea[2], E. Remenar[4], M. Degardin[5], I. S. Pateras[6], J. A. Langendijk[7], C. van Herpen[8], A. Awada[9], J. R. Germà-Lluch[10], H. R. Kienzer[11], L. Licitra[12] and J. B. Vermorken[13]

[1] Medicine, Attikon University Hospital, National and Kapodistrian University of Athens, Athens, Greece;

[2] EORTC Headquarters, Brussels, Belgium;

[3] Department of Biochemistry and Molecular Biology, Faculty of Biology, National and Kapodistrian University of Athens, Athens, Greece;

[4] Head & Neck Department, Országos Onkológiai Intézet, Budapest, Hungary;

[5] Oncology, Centre Oscar Lambret, Wattignies, France;

[6] Molecular Carcinogenesis Group, Lab Histology-Embryology, Medical School, National and Kapodistrian University of Athens, Athens, Greece;

[7] Department of Radiation Oncology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands;

[8] Department of Medical Oncology, Radboud University Medical Center, Nijmegen, Netherlands;

[9] Medical Oncology Clinic, Jules Bordet Institute, Université Libre de Bruxelles Bruxelles, Belgium;

[10] Medical Oncology, ICO L'Hospitalet - Hospital Duran i Reynals (Institut Catala D'Oncologia), L'Hospitalet de Llobregat, Spain;

[11] 3rd Med. Department, KFJ-Spital/SMZ Sud, Wien, Austria;

[12] Head and Neck Cancer Medical Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milan, and University of Milan, Milan, Italy;

[13] Medical Oncology, Antwerp University Hospital, Edegem, Belgium

Corresponding author: Dr. Amanda Psyrri, National and Kapodistrian University of Athens, School of Medicine, Second Department of Internal Medicine, Section of Medical Oncology, "Attikon" University Hospital, 124 61 Athens, Greece. Tel: +30-210-583-1256, Fax: +30-210-583-1690, E-mail: dpsyrri@med.uoa.gr

ABSTRACT

Background: EORTC 24971 was a phase III trial demonstrating superiority of induction regimen TPF over PF, in terms of progression-free (PFS) and overall survival (OS) in locoregionally advanced unresectable HNSCC. We conducted a retrospective analysis of prospectively collected data aiming to evaluate whether only HPV(−) patients (pts) benefit from adding docetaxel to PF, in which case de-intensifying induction treatment in HPV(+) pts could be considered.

Methods: Pre-therapy tumor biopsies (blocks or slides) were assessed for high-risk HPV by p16 immunohistochemistry, PCR and qPCR. HPV-DNA+ and/or p16+ tumors were subjected to *in situ* hybridization (ISH) and HPV *E6* oncogene expression qRT-PCR analysis. Primary and secondary objectives were to evaluate the value of HPV/p16 status as predictive factor of treatment benefit in terms of PFS and OS. The predictive effect was analyzed based on the model used in the primary analysis of the study with the addition of a treatment by marker interaction term and tested at two-sided 5% significance level.

Results: 119 of 358 pts had available tumor samples and 58 of them had oropharyngeal cancer. Median follow-up was 8.7 years. Sixteen of 119 (14%) evaluable samples were p16+ and 20 of 79 (25%) evaluable tumors were HPV-DNA+. 13 of 40 pts (33%) assessed with HPV-DNA ISH and 12 of 28 pts (43%) assessed for HPV *E6* mRNA were positive. The pre-planned analysis showed no statistical evidence of predictive value of HPV/p16 status for PFS (p=0.287) or OS (p=0.118).

Conclusions: The incidence of HPV positivity was low in the subset of EORTC 24971 pts analyzed. In this analysis only powered to detect a large treatment by marker interaction there was no statistical evidence that treatment effect found

overall was different in magnitude in HPV(+) or HPV(−) pts. These results do not justify selection of TPF versus PF according to HPV status.

Keywords

human papillomavirus; head and neck cancer; oropharyngeal cancer; EORTC 24971/TAX323 phase III clinical trial; TPF induction chemotherapy; HPV16

Key message

HPV status of locally advanced unresectable head and neck squamous cell carcinoma (HNSCC) patients do not justify selection of TPF to PF induction chemotherapy.

INTRODUCTION

Head and Neck Squamous Cell Carcinomas (HNSCC) is expected to account for approximately 740,000 new cases and 410,000 deaths worldwide, for the year 2015 [1]. In Europe, HNSCC incidence and mortality rates are higher compared to the United States, with approximately 140,000 new cases diagnosed in 2014, corresponding to an annual incidence of 43/100,000 [2]. Tobacco and alcohol use account for the vast majority of HNSCC. Human papillomavirus (HPV) infection has been more recently identified as the cause of a distinct subset of HNSCC that arise primarily in the oropharynx [3]. In particular, HPV16 is responsible for more than 90% of HPV positive (HPV(+)) OPSCC [4].

Accumulating evidence clearly suggests that HPV-associated OPSCC represent a distinct entity in terms of epidemiology, biology and clinical behavior. Multiple methods for determination of HPV status are available. HPV DNA detection in tumors *per se* cannot prove causal association as HPV is ubiquitously present in humans. HPV DNA PCR is a sensitive but not specific method for determination of HPV status. Immunohistochemistry (IHC) for p16 protein expression is used as a surrogate marker of HPV infection in OPSCC. A negative autoregulatory loop between p16 and pRb has been described [5] and degradation of pRb by HPV E7 oncoproteins leads to p16 upregulation in HPV positive cancers. p16 IHC followed by PCR for HPV DNA has been proposed as a reliable algorithm for detection of HPV in paraffin embedded OPSCC specimens. p16 protein expression, however, is not a reliable surrogate marker for HPV infection outside the oropharynx. HPV DNA *in situ* hybridization (ISH) can differentiate between integrated and episomal forms of HPV in tumors but lacks sensitivity. The gold standard is detection of E6/E7 mRNA, but this may be less sensitive depending on the quality of the clinical sample. In addition, many HPV(+) patients identified in the next generation sequencing study by Parfenov *et al*. had low expression or absence of E6/E7 expression and could be misclassified by E6/E7 mRNA detection [6].

HPV(+) OPSCC has a significantly better prognosis independent of stage at diagnosis compared to their HPV(−) counterpart [7]. The risk of death for HPV(+) patients is consistently less than 60% that of HPV(−) cancers across studies and the absolute survival difference is consistently higher than 30%. Deintensification research strategies that aim to reduce treatment-related morbidity and improve patient quality of life without compromising treatment effectiveness are being tested in clinical trials. Patients with HPV(+) OPSCC are young and expected to live long, therefore, morbidity resulting from late toxicity is a concern in these patients. To that extent, in the present study our aim was to evaluate whether only HPV(−) patients derive benefit from adding docetaxel to PF, in which case deintensifying induction treatment in HPV(+) patients could be considered.

This project was approved by the EORTC Translational Research Advisory Committee (TRAC) in May 2010.

MATERIALS AND METHODS

Subjects and Collection of biological material

EORTC 24971 randomized 358 patients with primary inoperable non metastatic HNSCC between TPF (docetaxel, cisplatin, 5-fluorouracil) chemotherapy and PF (cisplatin/5-fluorouracil) induction chemotherapy followed by radiotherapy and demonstrated statistically significant improvements in progression free survival (PFS) and overall survival (OS) with the addition of docetaxel to PF [8]. Samples have been collected in the frame of the EORTC 24971 study from 1999 until 2002.

The two main challenges to conduct the retrospective translational research (TR) study were: (1) to get a clear view on the available material – as the research was not planned at the time of the protocol; (2) to get Ethical Committees approval for this research while the patient information sheet/inform consent did not mention any kind of biological material collection (in the frame of planned nor unplanned/future research).

In order to solve the first point, feasibility was conducted among the participating sites, from 2009 until 2011. In 2011, 14 sites answered positively to the request (corresponding to 209 patients) and 5 sites answered negatively (e.g. tissue samples not available anymore). Regarding the second point, most of the ECs did not have any specific requests (patient reconsent, etc). In France, the initial EC feedback required a declaration of biological collection, with abstention of ministerial approvals and patients reconsent/special derogation for deceased patients. Finally, the collection was possible as the EC agreed to the collection for deceased or lost in follow-up patients, without derogations and without ministerial approvals. In total, 4 years were dedicated to the operational part of setting-up this translational research project, covering the feasibility, the EC submission and approval processes, and the sample shipment.

We managed to collect 119 HNSCC tissue samples which were assessed for high risk HPV infection by p16 IHC, PCR and quantitative PCR (qPCR). HPV DNA+ and/or p16+ tumors were subjected to ISH and HPV *E6* oncogene expression analysis by qRT-PCR.

Assays

Immunohistochemical (IHC) staining for p16

IHC was performed to determine p16 expression using a p16 mouse monoclonal antibody (predilute, mtm-CINtech, E6H4) as previously described [9]. p16 was considered to be positive when defined as strong and diffuse nuclear and cytoplasmic staining in ≥70% of the tumor cells, which is the same scoring criteria used by Ang *et al.* [9].

DNA extraction from paraffin sections

Two 10 µm-paraffin sections from HNSCC specimens were used for DNA extraction by the QIAamp DNA FFPE Tissue kit (QIAGEN, Germany), according to

manufacturer's instructions. The DNA was eluted in QIAGEN ATE buffer and stored at -20^{o}C until analysis.

Detection of high-risk HPV DNA by PCR

For high-risk HPV DNA detection, the two most popular worldwide consensus PCR reactions were used: the MY system [10] and the GP+ system [11] both amplifying regions of L1 HPV gene. DNA integrity was assessed by PCR amplification of *β-globin* with PC04 and GH020 primers [10]. PCR reactions were performed in the GENEAmp PCR System 9600 (Applied Biosystems, USA). The protocols for the PCR detection of high-risk HPV DNA are provided in supplementary information (SI) text.

HPV RFLP typing

In case of a positive sample in the MY and/or GP+ PCR system, reactions were performed again in quadruplicate, mixed and their product was subjected to restriction fragment polymorphism analysis using the BamHI, DdeI, HaeIII, HinfI, PstI and RsaI restriction enzymes (New England Biolabs, USA). Analysis performed in 2% Nusieve 1:1 agarose gel as previously reported [12]. Assignment of an HPV type to a particular risk category was done according to Munoz *et al*. [13]. The protocols for HPV RGLP typing are provided in SI text.

Real time qPCR for detection of HPV16, 18 and 31

In case of negative samples in MY PCR system, novel real-time qPCR assays were developed in order to provide a more sensitive detection for those HPV types most commonly found in oropharyngeal cancers; types HPV16, 18 and 31. The qPCR primers used amplify a 93bp HPV16 E6 region [14], a 185bp HPV18 E1 region [15] and a 350bp HPV31 E6 region (*in-house assay*). The protocols for real-time qPCR detection of HPV16, 18 and 31 are provided in SI text.

RNA extraction from hematoxylin and eosin stained slides

RNA was extracted from hematoxylin and eosin stained slides using High Pure FFPET RNA Isolation Kit (ROCHE, Germany), according to manufacturer's instructions. The RNA was eluted in ROCHE Elution buffer and stored at -80^{o}C until analysis.

cDNA synthesis

cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Life technologies, USA) according to manufacturer's protocol. Additional information for cDNA synthesis is provided in SI text.

HPV *in situ* hybridization

High-risk HPV status was determined by ISH using a cocktail probe that detects HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (GenPoint HPV probe cocktail; Dako, Carpinteria, CA). HPV ISH was interpreted as positive when nuclear-specific staining was detected in the tumor cells. The protocols for HPV ISH are provided in SI text.

Detection of HPV E6 mRNA on paraffin embedded tissue

Novel SYBR Green-based qPCR assays were developed for the detection of E6 mRNA expression of HPV16, 18 and 31 on FFPE samples. The specific qPCR primers used give rise to a 109bp HPV16-specific and a 131bp HPV18-specific amplicons, as previously described [16, 17]. Novel specific primers for HPV31 E6 gene expression were designed giving rise to a 165bp specific amplicon. *GAPDH* and *β-actin* were used as our assays endogenous reference genes. The protocols for the real-time qPCR detection of HPV16, 18 and 31 E6 expression are provided in SI text.

Statistical methods

PFS and OS curves by treatment and marker strata were produced using the Kaplan-Meier method. PFS and OS were based on the long-term survival analysis produced in 2011 [18]. Statistical significance of predictive effects was assessed based on the model used in the primary analysis of the study with the addition of a treatment by marker interaction term. This was a multivariate Cox proportional hazards model with treatment, marker and treatment by marker interaction effects, adjusted for the following covariates: location of primary tumor (oral cavity, oropharynx, or hypopharynx), clinical tumor stage (T), regional-node stage (N), and WHO score for performance status. For the two initially planned analyses, the treatment by marker interaction was tested at a two-sided 5% significance level. The estimate of the hazard ratio HR for treatment in each marker strata was provided with its 95% confidence interval. For the additional analyses (22 statistical tests), the Benjamini-Hochberg method was applied to control the False Discovery Rate (FDR). Additional information is provided in SI text.

RESULTS

Patient Population

A total 119 tissue samples were obtained from 119 of the 358 patients included in EORTC 24971 study and assessed for p16/HPV markers. Eighty two samples were FFPE blocks and 37 were FFPE slides; Median follow-up for these 119 patients was 8.7 years. The baseline characteristics of these patients are shown in supplementary table S1. Patients selected for this project did not differ significantly from the rest of patients with respect to age, gender, tumor site, T stage, nodal stage, although baseline performance status was better, as assessed by t-test for age and from Fisher's exact test for binary or categorical variables. Kaplan-Meier curves of PFS and OS by treatment arm for the 119 patients are shown in Figure 1.

p16 Immunohistochemistry

One hundred nineteen specimens were evaluable for p16 by immunohistochemistry. Sixteen of the 119 patients were positive for p16: The incidence of p16 positivity in the cohort was 13.4% (95% CI: 7.9%-20.9%). The distribution of p16 positivity by tumor site is shown in Table 1.

Detection of high-risk HPV DNA by PCR

Seventy nine of 82 FFPE were evaluable for HPV DNA by PCR. All samples were subject to histopathological evaluation, DNA quality control, and HPV DNA detection. Twenty of 79 FFPE (25.3%, 95% CI: 16.2%-36.4%) specimens were positive for high-risk HPV DNA (Table 1). Of the 20 HPV(+) FFPEs samples, 8 contained HPV16, one HPV18, 7 HPV31 and 3 had infection with both HPV16 and HPV31, while one was unspecified. Ten of 20 HPV DNA+ FFPEs by PCR were also p16+. The distributions of HPV DNA by p16 status and of p16/HPV DNA positive samples by tumor site are provided in supplementary tables S2 and S3, respectively.

HPV in situ hybridization

HPV ISH using a set of probes for high-risk HPV detection was performed both in cases that had slides instead of FFPEs as well as in cases with positive status for either p16 or HPV DNA by PCR. Forty one samples met these criteria and 13 of those were HPV(+) by ISH (32.5%, 95% CI: 18.6%-49.1%) (Table 1).

Detection of HPV E6 mRNA on FFPE

HPV DNA+ and/or p16+ tumors were subjected to HPV *E6* oncogene expression analysis by qRT-PCR. Twenty eight samples were evaluable for high-risk HPV RNA analysis by qRT-PCR and 12 of them (11 for HPV16 and 1 for HPV18) were positive for HPV *E6* mRNA (42.9%, 95% CI: 24.5%-62.8%).

Predictive effect of HPV status

The pre-planned analysis showed no statistical evidence of predictive value of p16/HPV DNA by PCR status for PFS (p=0.287) or OS (p=0.118). Kaplan-Meier curves are shown in Figure 1.

The additional analyses, adjusted for multiplicity to control FDR, showed no statistical evidence of a prognostic or predictive effect; on PFS or OS; based on either markers; in all tumors or restricted to oropharynx.

DISCUSSION

In the present study we sought to evaluate whether only HPV(−) patients derive benefit from adding docetaxel to PF in induction chemotherapy, in which case deintensifying induction treatment in HPV(+) patients could be considered. We analyzed pretreatment specimens from patients enrolled in EORTC 24971, a phase III study demonstrating superiority of induction regimen TPF over PF in terms of progression-free (PFS) and overall survival (OS) in locoregionally advanced unresectable HNSCC [8], for HPV status. The magnitude of the treatment effect found overall was not statistically different in p16/HPV positive or negative patients. However, this was a prospective - retrospective analysis only powered to detect a large treatment by marker interaction.

Our primary and secondary objectives were to evaluate the value of HPV/p16 status as predictive factor of treatment benefit in terms of PFS and OS, respectively. The study was designed in 2008 and at that time p16 immunohistochemistry followed by HPV DNA PCR in p16 positive cases had been demonstrated to be a reliable algorithm for a biologically and clinically relevant HPV infection in OPSCC. We therefore presumed that the same algorithm could be used for HPV detection in non-OPSCC. However, it was subsequently shown that p16 protein expression is not a reliable surrogate marker for HPV infection outside the oropharynx [19]. Our study

population consisted of both OPSCC and non-OPSCC. We therefore performed HPV ISH and HPV E6 expression analysis in HPV DNA+ and/or p16+ cases. We found that the incidence of HPV was low in our EORTC 24971 cohort. The study was conducted between April 1999 and March 2002 and our findings are consistent with other studies examining the incidence of HPV infection in European populations during the same time period [20-23].

We found that 8 of 20 HPV DNA+ FFPEs contained HPV16, one HPV18, 7 HPV31, 3 had infection with both HPV16 and HPV31 and one was unspecified. Of the 28 HPV DNA+ and/or p16+ samples analyzed, expression of *E6* oncogene detected in 12 samples, of which 11 samples were positive for HPV16 *E6* and 1 sample positive for HPV18 *E6* expression. In the study by Bratman *et al*. [24], the presence of HPV was determined for 515 HNSCCs from TCGA. Seventy-three tumors contained HPV transcripts, among which 61 (84%) were HPV16, and 12 (16%) were HPV-other (8 HPV33, 3 HPV35, and 1 HPV56). The authors reported that HNSCCs harboring HPV genotypes other than HPV16 have inferior survival. However, p16 status was not available for all cases with HPV-other genotypes; a prospective study of HPV-other genotype in OPSCC, uniformly characterized with p16 staining and tobacco history, and uniformly treated, is required before concluding that HPV-other genotypes confer poor prognosis in HNSCC.

In addition to oropharynx, the subsite for which HPV carcinogenesis is best understood, our study also included oral cavity, larynx and hypopharynx. In the study of 3680 cases of HNSCC by Castellsagué *et al*. [25] the incidence of HPV positivity was 22.4%, 4.4%, and 3.5% for cancers of the oropharynx, oral cavity, and larynx, respectively. HPV16 was the dominant genotype. In our study the incidence of HPV/p16 double positive was 6/10, 1/22, 1/7 and 2/32 in oropharynx, oral cavity, larynx and hypopharynx respectively.

Our retrospective study was only powered to detect a large treatment by marker interaction. The study data did not show any evidence that HPV(+) or HPV(−)

patients benefit more from the addition of docetaxel to PF. Similar conclusion was reached with the HPV analysis of TAX324 study, a phase III study comparing TPF to PF in patients with locoregionally advanced HNSCC treated with sequential therapy [26]. In this unplanned analysis of survival involving 111 patients, the authors reported no statistical differences between TPF and PF in HPV positive and HPV negative patients and that was attributed to the small numbers of patients and concomitant loss of statistical power.

In conclusion, our study found that the incidence of HPV positivity in a subset of patients enrolled in EORTC 24971 was low. In this prospective-retrospective analysis only powered to detect a large treatment by marker interaction there was no statistical evidence that treatment effect found overall was different in magnitude in HPV(+) or HPV(−) negative patients. These results do not justify selection of TPF versus PF according to HPV status.

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Figure legends

Figure 1. Kaplan-Meier curves for PFS and OS for the subset of patients included in this project, by treatment arm (A, B) and by by treatment arm and p16 / HPV DNA by PCR status (C, D).

Table 1: Distribution of p16/HPV by tumor site

p16/HPV by tumor site					
	primary tumor site				
	Oral cavity (*N*=22)	Oropharynx (*N*=58)	Hypopharynx (*N*=32)	Larynx (*N*=7)	Total (*N*=119)
	N (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
IHC for p16					
negative	19 (86.4)	49 (84.5)	29 (90.6)	6 (85.7)	103 (86.6)
positive	3 (13.6)	9 (15.5)	3 (9.4)	1 (14.3)	16 (13.4)
PCR for HPV DNA[1]					
negative	7 (31.8)	27 (46.6)	19 (59.4)	6 (85.7)	59 (49.6)
positive	1 (4.5)	13 (22.4)	5 (15.6)	1 (14.3)	20 (16.8)
non evaluable	1 (4.5)	2 (3.4)	0 (0.0)	0 (0.0)	3 (2.5)
not done	13 (59.1)	16 (27.6)	8 (25.0)	0 (0.0)	37 (31.1)
HPV subtypes (only for HPV DNA positive samples)					
unspecified	0 (0.0)	0 (0.0)	1 (3.1)	0 (0.0)	1 (0.8)
type 16	0 (0.0)	6 (10.3)	2 (6.3)	0 (0.0)	8 (6.7)
type 18	0 (0.0)	1 (1.7)	0 (0.0)	0 (0.0)	1 (0.8)
type 31	1 (4.5)	4 (6.9)	2 (6.3)	0 (0.0)	7 (5.9)
type 16 combined with type 31	0 (0.0)	2 (3.4)	0 (0.0)	1 (14.3)	3 (2.5)
missing	21 (95.5)	45 (77.6)	27 (84.4)	6 (85.7)	99 (83.2)
ISH for HPV DNA[2]					
HPV DNA ISH negative	2 (9.1)	18 (31.0)	5 (15.6)	2 (28.6)	27 (22.7)
HPV DNA ISH positive	4 (18.2)	4 (6.9)	3 (9.4)	2 (28.6)	13 (10.9)
not evaluable / not done	16 (72.7)	36 (62.1)	24 (75.0)	3 (42.9)	79 (66.4)
PCR for HPV RNA					
HPV RNA PCR negative	3 (13.6)	8 (13.8)	4 (12.5)	1 (14.3)	16 (13.4)
HPV RNA PCR positive	2 (9.1)	8 (13.8)	1 (3.1)	1 (14.3)	12 (10.1)
not evaluable / not done[3]	17 (77.3)	42 (72.4)	27 (84.4)	5 (71.4)	91 (76.5)
HPV subtypes (only for HPV RNA positive samples)					
type 16	2 (9.1)	7 (12.1)	1 (3.1)	1 (14.3)	11 (9.2)
type 18	0 (0.0)	1 (1.7)	0 (0.0)	0 (0.0)	1 (0.8)
missing	20 (90.9)	50 (86.2)	31 (96.9)	6 (85.7)	107 (89.9)

[1] PCR for HPV DNA was assessed in 82 FFPE blocks
[2] HPV ISH was performed both in cases that had slides instead of FFPEs as well as in cases with positive status for either p16 or HPV DNA by PCR (40 samples)
[3] FFPE samples were often not of good quality for RNA analysis

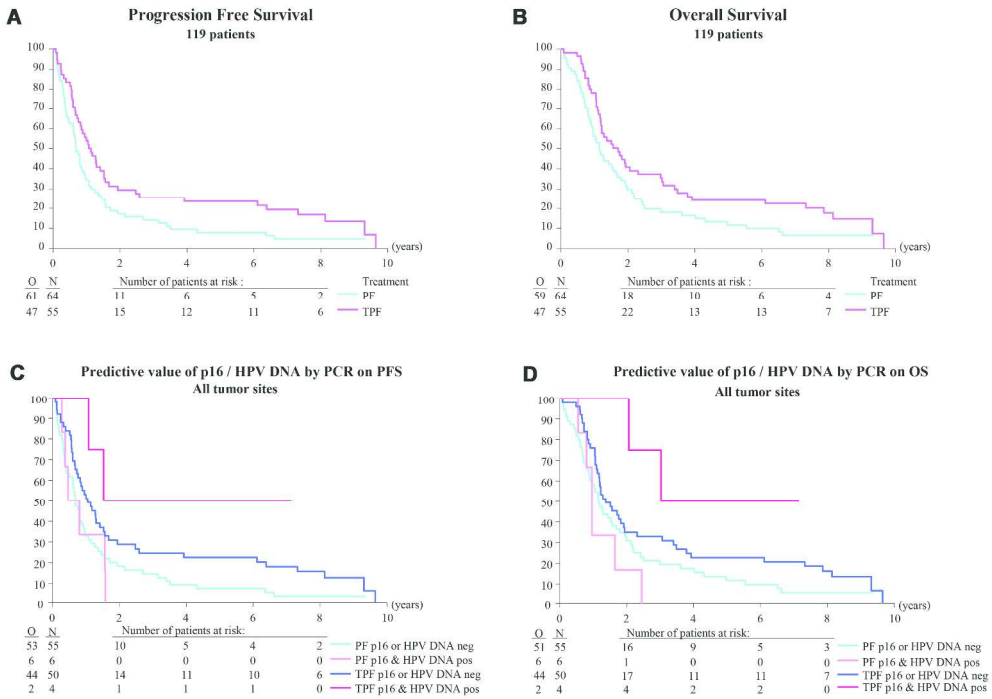

Figure 1. Kaplan-Meier curves for PFS and OS for the subset of patients included in this project, by treatment arm (A, B) and by by treatment arm and p16 / HPV DNA by PCR status (C, D).

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SUPPLEMENTARY INFORMATION

Title: Evaluation of the impact of tumor HPV status on outcome in patients with locally advanced unresectable head and neck squamous cell carcinoma (HNSCC) receiving cisplatin, 5-fluorouracil with or without docetaxel: A subset analysis of EORTC 24971 study

Authors: A. Psyrri, C. Fortpied, G. Koutsodontis, M. Avgeris, C. Kroupis, N. Goutas, J. Menis, L. Herman, L. Giurgea, E. Remenar, M. Degardin, I. S. Pateras, J. A. Langendijk, C. van Herpen, A. Awada, J. R. Germà-Lluch, H. R. Kienzer, L. Licitra and J. B. Vermorken

MATERIALS AND METHODS

Assays

Detection of high-risk HPV DNA by PCR

For high-risk HPV DNA detection, the two most popular worldwide consensus PCR reactions were used: the MY system [1] and the GP+ system [2] both amplifying regions of L1 HPV gene. DNA integrity was assessed by PCR amplification of *β-globin* with PC04 and GH020 primers [1]. PCR reactions were performed in the GENEAmp PCR System 9600 (Applied Biosystems, USA).

PCR reactions were performed in a 25 μl total reaction volume, containing 50-100 ng genomic DNA, 2.5 μl of 10X PCR buffer (w/o $MgCl_2$), 3 μl of 10 mM dNTPs mix, 0.75 μl of 50 mM $MgCl_2$, 1.75 μl of 10 μM forward primer, 1.75 μl of 10 μM reverse primer and 0.25 μl of 5 U/μl Platinum Taq DNA polymerase (Invitrogen, Life Technologies). Amplification for *β-globin* was performed with the following cycling profile: incubation at 94 °C for 5 min followed by 36 cycles of 45 sec denaturation at 94 °C, 45 sec annealing at 58 °C and 45 sec elongation at 72°C. The last cycle was followed by a final extension of 5 min at 72°C. Amplification for MY and GP+ systems

was performed with the following cycling profile: incubation at 94 °C for 5 min followed by 40 cycles of 2 min denaturation at 94 °C, 2 min annealing at 55 °C for MY and 40°C for GP+ and 2 min elongation at 72°C. The last cycle was followed by a final extension of 5 min at 72°C. Appropriate controls including DEPC-H_2O (blank reaction), DNA-negative for HPV and DNA-positive for HPV16 from SiHa cervical carcinoma cell line, were used. All necessary standard precautions were observed in order to avoid contamination through PCR carry-over. PCR products were analyzed in 1.5% w/v 1X TBE agarose gels.

HPV RFLP typing

In case of a positive sample in the MY and/or GP+ PCR system, reactions were performed again in quadruplicate, mixed and their product was subjected to restriction fragment polymorphism analysis: 13 μl of PCR product plus 1.5 μl restriction buffer NEB 2 and 0.5 μl of each of these restriction enzymes: BamHI, DdeI, HaeIII, HinfI, PstI and RsaI (New England Biolabs, USA) in separate tubes. Incubations lasted 4 h at 37°C and were analyzed subsequently in a 2% Nusieve 1:1 agarose gel as previously reported [3]. Assignment of an HPV type to a particular risk category was done according to Munoz *et al*. [4].

Real time qPCR for detection of HPV16, 18 and 31

In case of negative samples in the MY PCR system, novel real-time qPCR methods were developed in order to provide a more sensitive detection for those HPV types most commonly found in oropharyngeal cancers; types 16, 18 and 31. The primers for types 16 and 18 have been described elsewhere and amplify a 93bp for HPV16 E6 region and a 185bp for HPV18 E1 region, respectively [5, 6]. The following primers were synthesized in order to amplify a 350bp E6 region of HPV31 type: HPV31F: 5'-TAAGCTCGGCATTGGAAATACCCT-3' and HPV31R: 5'-CCTTCCTCCTATGTTGTGGAATCG-3'. The assays for HPV16 and HPV18 were

calibrated against the WHO international certified reference materials from NIBSC (UK) and they were able to easily detect a viral load of 5 IU/μl from each of the two types. The assay for HPV31 was calibrated against an *in-house* HPV31+ cervical carcinoma sample.

The qPCR reactions were performed in glass capillaries in the LightCycler platform (Roche Applied Science) in a 10 μl total reaction volume, containing 50-100 ng genomic DNA, 1 μl of 10X DNA Master SYBR Green I reagent (Roche Applied Science), 0.2 μl of 20 μM forward primer and 0.20 μl of 20 μM reverse primer. Amplification was initiated with an incubation at 94 °C for 5 min followed by 45 cycles of 10 sec denaturation at 95 °C, 30 sec annealing at 57 °C (for HPV16 at 60 °C) and 40 sec elongation at 72°C. The last cycle was followed by melting curve analysis (10 sec denaturation at 95 °C, 30 sec at 50 °C and up to 95°C at a 0.1 °C/sec rate with continuous monitoring of the F1 channel) for verification of the specificity of the reactions. The Tm of the amplicons was 79.07±0.48, 81.31±0.07 and 78.34±0.47 °C (average ± SD) for types 16, 18 and 31 respectively.

cDNA synthesis

cDNA synthesis was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Life technologies, USA) according to manufacturer's protocol. 7μl of isolated total RNA was used as starting template. Appropriate controls, DEPC-H_2O (blank reaction) and positive for HPV16 RNA from SiHa cervical carcinoma cell line, were included. All necessary standard precautions were observed in order to avoid contamination through PCR carry-over.

HPV *in situ* hybridization

High-risk HPV status was determined by ISH using a cocktail probe that detects HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 (GenPoint HPV probe cocktail; Dako, Carpinteria, CA). HPV ISH was interpreted as positive when

nuclear-specific staining was detected in the tumor cells. In brief, sections after deparaffinization and rehydration, were pretreated with Target Retrieval solution for 30 minutes, followed by background quenching with 0.3% H_2O_2 in methanol. Probe and target DNA denaturation was performed by heating at 92°C for 5 minutes and hybridization took place at 37°C overnight. Stringent wash step took place at 48°C for 30 minutes. Detection of hybridized probe was performed by successive incubation of slides with Primary Streptavidin-HRP, followed by biotinyl tyramide and secondary Streptavidin-HRP solution. Finally sections were stained with DAB and counterstained with hematoxylin. Enzymatic reaction results in visible dark blue/black dots in the cell nuclei of positive cases. Cores from HPV16+ SiHa and HPV18+ HeLa cervical cancer cell lines fixed in formalin and embedded in paraffin were selected for positive controls. Evaluation of the HPV status was performed by two independent pathologists (N.G. and I.S.P.) under light microscopy.

Detection of HPV E6 mRNA on paraffin embedded tissue

Novel SYBR Green-based qPCR assays were developed and applied for the detection of E6 mRNA expression of HPV types 16, 18 and 31 on FFPE samples. Specific primers for HPV16 and HPV18 E6 genes were used as described previously in Shi *et al*. [7] (HPV16 E6 forward 5'-CAGTTATGCACAGAGCTGCAA-3' and HPV16 E6 reverse 5'-AATCCCGAAAAGCAAAGTCAT-3';) and Jha *et al*. [8] (HPV18 E6 forward 5'-CCAGAAACCGTTGAATCCAG-3' and HPV18 E6 reverse 5'-GTTGGAGTCGTTCCTGTCGT-3';), amplifying a 109 bp and a 131 bp specific products of HPV16 and HPV18 E6 mRNA, respectively. Novel specific primers for the detection of HPV31 E6 gene expression (HPV31 E6 forward 5'-CCTGCAGAAAGACCTCGGAA-3' and reverse 5'-TGGTGTGTCGTCCCTATATACTATTG-3') were designed giving rise to a 165 bp specific amplicon. *GAPDH* and *β-actin* were used as our assays endogenous reference genes. Specific *β-actin*, as described in Shi *et al*. [7] (*β-actin* forward 5'-

CCCAGATCATGTTTGAGACCT-3' and *β-actin* reverse 5'-AGTCCATCACGATGCCAGT-3';), and *GAPDH* (*GAPDH* forward 5'-ATGGGGAAGGTGAAGGTCG-3' and *GAPDH* reverse 5'-GGGTCATTGATGGCAACAATATC-3') primers were synthesized and used for the amplification of 105 bp and 107 bp specific products, respectively.

The qPCR was performed in the 7500 Real-Time PCR System using the sequence detection software (Applied Biosystems, Carlsbad, CA, USA). The 10 µl reaction mixture consists of Kapa SYBR® Fast Universal 2X qPCR Master Mix (Kapa Biosystems, Inc., Woburn, MA, USA), 300 nM of each qPCR primer and 1.0 µl cDNA for HPV16 E6, *GAPDH* and *β-actin* reactions, or 1.5 µl cDNA for HPV18 E6 and HPV31 E6 reactions. The thermal protocol consists of a 3 min polymerase activation step at 95^{o}C, followed by 40 cycles of denaturation at 95^{o}C for 15 sec and the primer annealing and extension step at 60^{o}C for 1 min. Melting curve analysis and agarose gel electrophoresis were performed following the amplification in order to distinguish the accumulation of the specific reaction products from non-specific ones or primer-dimers.

Duplicate reactions were performed for each tested sample and the average C_T was calculated and evaluated for the analysis. Both *GAPDH* and *β-actin* were used as our assays endogenous reference genes for normalization purposes. SiHa cervix squamous cell carcinoma cell line and HeLa cervix adenocarcinoma cell line were used as our assays positive control for HPV16 and HPV18 E6 mRNA, respectively, while for HPV31 we used an *in-house* HPV31+ cervical carcinoma sample.

Statistical methods

The primary and secondary objectives were to evaluate the value of HPV/p16 status as predictive factor of treatment benefit in terms of PFS and OS respectively, in patients who participated in the EORTC 24971/TAX323 phase III clinical trial.

Additional investigations were proposed in a second stage to evaluate (1) the prognostic effect of p16/HPV markers; (2) the predictive and prognostic effect of HPV DNA ISH and HPV RNA qPCR. In addition, the same analysis, restricted to oropharynx only, was conducted. The following markers were assessed in these samples: a. Conventional IHC on TMA for p16 expression status (positive/negative), b. PCR and qPCR for HPV DNA (positive/negative), c. ISH for HPV DNA (positive/negative), d. qPCR for HPV E6 mRNA (positive/negative). The first two (a. and b.) were combined in order to classify patients into two groups: p16 or HPV DNA PCR negative and p16 & HPV DNA PCR both positive.

PFS and OS curves by treatment and marker strata were produced using the Kaplan-Meier method. PFS and OS were based on the long-term survival analysis produced in 2011 [9]. Statistical significance of predictive effects was assessed based on the model used in the primary analysis of the study with the addition of a treatment by marker interaction term. This was a multivariate Cox proportional hazards model with treatment, marker and treatment by marker interaction effects, adjusted for the following covariates: location of primary tumor (oral cavity, oropharynx, or hypopharynx), clinical tumor stage (T), regional-node stage (N), and WHO score for performance status. For the two initially planned analyses, the treatment by marker interaction was tested at a two-sided 5% significance level. The estimate of the hazard ratio HR for treatment in each marker strata was provided with its 95% confidence interval. For the additional analyses (22 statistical tests), the Benjamini-Hochberg method was applied to control the False Discovery Rate (FDR). Additional information is provided in supplementary information text.

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